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## Ultrastructural pathology Associated with Attachment of *Pasteurella multocida* serotype B: 2 to Tracheal and Pulmonary Endothelial Cells and Tracheal cilia of Mice

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### Abstract

Mice infected intraperitoneally with *P. multocida* at the dose of  $10^7$  cells showed trachea with loss of cilia. The cilia become deformed with electron dense outline and bacterial cells were seen attached to the deformed cilia. Many bacteria were also seen closely attached to endothelial cells causing several ultrastructural changes at the sites of attachment. The cytoplasm at the site of bacterial attachment appeared to extend into either short or long pedastal (attaching and effacing lesions) into the blood vessel lumen with a single bacteria attached to the pedastal. These pedastals are either electron dense or electron dense and vesicular. Occasionally, beneath these cytoplasmic extensions, focal increased in electron density of the cytoplasm were observed. Also, there were long thin, single cytoplasmic extensions (microvillus-like protrusions) around the attached bacteria. Invagination with two fingers like cytoplasmic extensions which appears to engulf the attaching bacteria were also seen. The cell membrane of some endothelial cell adjacent to the attached bacteria appeared depressed / invaginated.

**Keywords:** Pulmonary Endothelial Cells, *Pasteurella multocida* and Tracheal cilia

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### 1. Introduction

*Pasteurella multocida* is a Gram-negative, non-motile, fermentative, facultative anaerobic coccobacilli or rods (Kilian and Fredericksen, 1981). Certain serological types are the causing agents of severe pasteurellosis, such as hemorrhagic septicemia of cattle and water buffaloes in certain enzootic areas of Asia and Africa (Carter and DeAlwis, 1989). Haemorrhagic septicaemia is a highly fatal disease of cattle and water buffaloes. In susceptible animals, the symptoms progress rapidly from dullness and fever to death within hours. At necropsy, hemorrhages and oedema

are observed on serous and mucous surfaces, in lymph nodes, spleen, lungs and other organs (Carter, 1998).

Adherence to mucosal surfaces is recognized as an important step in colonization and infection (Smith, 1977). A number of infectious diseases are initiated by bacterial attachment and later colonization of the mucosal surfaces (Beachey, 1981). Many studies on the ultrastructural changes to host cell surfaces associated with bacterial adhesion and the bacterial organelles involved in the attachment process have been conducted (Ofeck and Doyle, 1994).

Some bacteria have evolved mechanisms for entering host cell by attaching to the host cell surface and causing changes in the host cell cytoskeleton. These changes involve polymerization and depolymerization of actin which occur as an integral part of pseudopod formation and engulfment of the bacteria in a phagocytic vesicle (Salyers and Whitt, 2005). Dramatic localized polymerization of actin and myosin at bacterial attachment sites seen as membrane ruffling in the vicinity of the invading bacteria have been reported in *Shigella flexneri* (Adam et al., 1995). This localized actin polymerization into filaments causes pseudopod extension and engulfment of *Shigella flexneri* which results in internalization of the pathogen within a vacuole (Adam et al., 1995). Epithelial cell surfaces, at sites where *Salmonella typhimurium* attached, developed increase in electron density that represent cytoskeletal microfilaments (Bilska et al., 1993). Ultrastructurally, these densities are represented as tiny granular foci that appear and disappear in concert with movement of bacteria into the cytoplasm (Bilska et al., 1993). The attachment of Enteropathogenic *E. coli* (EPEC) and enterohaemorrhagic *E. coli* (EHEC) to host cells form characteristic lesions called actin pedestals (attaching and effacing lesion) which are electron dense (Salyers and Whitt, 2005).

The invasion of epithelial cells by *P. multocida* serotype A was first detected by Al-Haddawi et al., (1999). The organism was observed to cause deformity to the cilia of the nasal epithelium (Al-Haddawi et al., 2000) and within vacuoles inside the cytoplasm of the nasal mucosal epithelium and type 1 pneumocyte of infected rabbits. The invasion of *P. multocida* results in nasal and pulmonary lesions in rabbits (Al-Haddawi et al., 1999). In contrary, Glorioso *et al.* (1982) showed that, ultrastructurally, *P. multocida* were associated with the surface cell of the pharyngeal mucosa but were not observed in the deeper layers of the epithelium. These observations have been made from *P. multocida* serotype A:3 isolated from rabbits. However, there have been insufficient report on the lesions that may develop when *P. multocida* serotype B: 2 interact with the host cell in the respiratory system. The suitability of using mice to study HS was suggested by Ramdani (1990). In this study we report the ultrastructural pathology associated with the adherence of *P. multocida* B: 2 to the trachea and pulmonary endothelial cells and tracheal cilia in mice.

## 2. Materials and Methods

### 2.1. Animals

Forty, eight to ten weeks old BALB/c mice of either sex were obtained from the Animal Resource Centre, Universiti Putra Malaysia. They were negative for *P. multocida* (4 days interval) of peripheral blood culture. They were housed in plastic cages and provided with water and pellet *adlibitum*. All the challenged experiments were performed in an isolated experimental room.

### 2.2 *Pasteurella multocida* Inoculum

*Pasteurella multocida* used in present study was stock culture of *P. multocida* B:2, isolated from previous outbreak of (HS) in the state of Kelantan, Malaysia. Identification of *P. multocida* was made using the Gram-staining method and biochemical characterization using the oxidase, urea broth, sulphur indole motility (SIM), triple sugar iron (TSI) and citrate test as described by Carter (1990). Pure culture was stored on nutrient agar slants and subcultured into 5% horse blood agar. A single colony of *P. multocida* was grown in brain heart infusion broth (BHI). The bacteria were quantitated by a ten-fold serial dilution in sterile phosphate buffered saline (PBS). Each mouse from infected group was inoculated intraperitoneally (i.p) with 0.1ml broth containing  $10^7$  cells. The control group mice were inoculated with 0.1 ml sterile PBS intraperitoneally.

### 2.3 Experimental design

Forty, mice were divided into 5 equal groups of eight mice each. Mice in groups 2, 3, 4, and 5 were inoculated intraperitoneally with *P. multocida* at the dose of  $10^7$  cells in each 0.1ml of PBS, while those in group 1 were inoculated with PBS, pH 7.4 and acted as control. Mice were observed for 24 hours to study the mortality pattern including the time of death. Twelve hours post infection, mice in group 2 were killed by cervical dislocation. Mice in groups 3, 4 and 5, died at 18, 20, 22 and 24 hours after infection.

## 2.4 Bacteriology

Blood swabs from the heart and samples from lung and aorta tissues were immediately cultured onto blood agar (containing 5% horse blood) and MacConkey agar and incubated at 37°C for 24 hours.

## 2.5 Bacterial isolation

*Pasteurella multocida* was isolated in pure culture from the swabs of heart blood, tracheal mucosa, lung tissue and aorta samples of dead mice in all infected groups.

## 2.6 Pathology

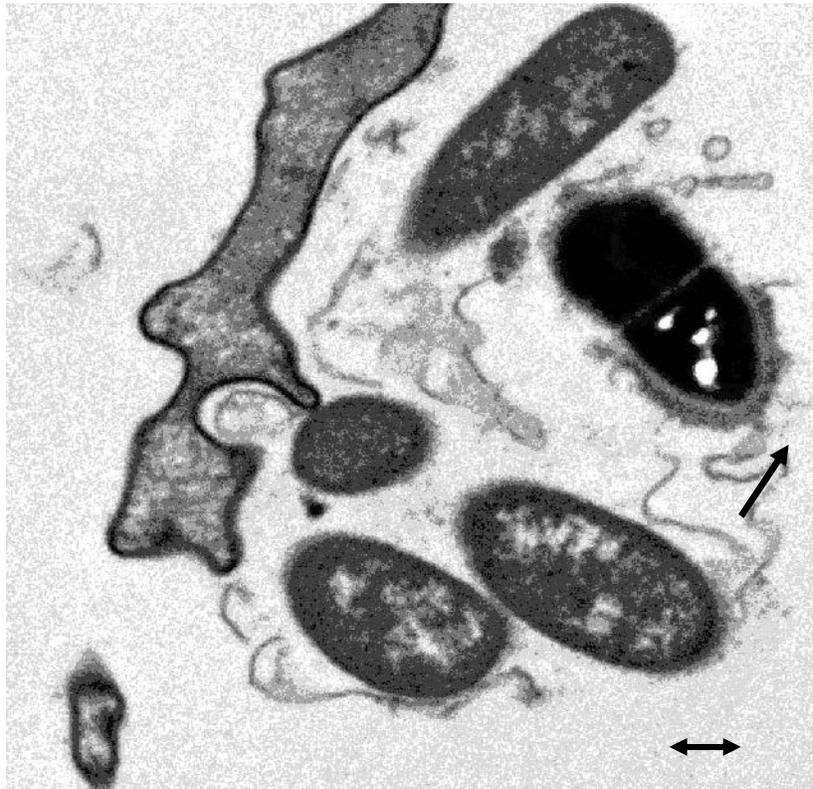
Dead mice were subjected to postmortem examination. For electron microscopic examination, 1mm<sup>3</sup> pieces of tissues of trachea and lung were fixed in 4% glutaraldehyde in 0.1M sodium cacodylate buffer (PH 7.4) at 4°C overnight. The samples were then washed at 10 minutes intervals with two changes of cacodylate buffer alone and processed for transmission electron microscopy (TEM) using standard methods. Briefly, the samples were fixed with 4% glutaraldehyde. The samples were then washed 3 times in 0.1 M sodium cocodylate buffer for 10 minutes each and post fixed for 2 hours with 1% osmium tetroxide (OsO<sub>4</sub>). Fixed samples were rinsed, dehydrated in a graded acetone and the samples were embedded in resins, cut into ultrathin sections on Ultracut E (Reichert-Jung, Austria) microtome and mounted on 200-mesh-copper grids. The sections were stained with uranyl acetate and lead citrate and examined using transmission electron microscope (Hitachi 7100, Japan)

## 3. Results

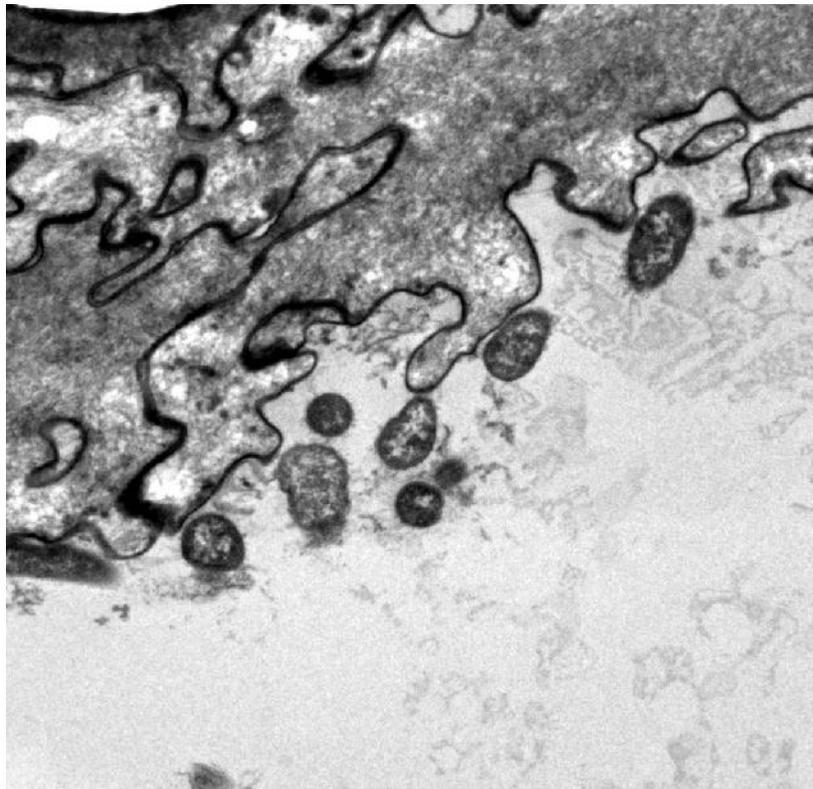
### 3.1 Trachea

In the trachea, there were cilia loss and cilia deformity. Bacterial cells were seen attached to the deformed cilia (Figures 1 and 2). The endothelial cells of the capillaries of the trachea of all infected mice were swollen with condensed cytoplasm. Necrotic endothelial cells were also seen protruded into the lumen of the affected blood vessel. Bacteria were evident among erythrocytes and platelets in the blood vessel lumina.

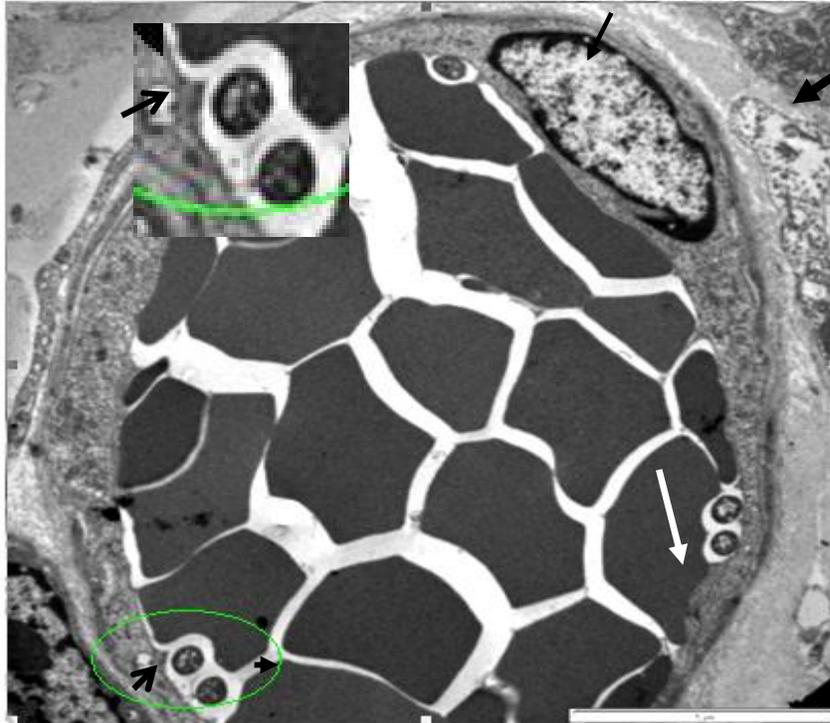
Erythrocytes filled the lumen and some were observed adhered to the endothelial cells. Bacteria in the capillary lumen were seen in close association with the plasma membrane of swollen endothelial cells. Large halos around these bacteria were observed. Several bacteria were seen attached to the plasma membrane of the endothelial cells. The cytoplasm of the endothelial cells was seen to extend into the lumen, like two short cytoplasmic extensions (microvillus-like protrusions) which appeared to engulf the bacteria (Figure 3). A single long cytoplasmic extension was also observed to engulf the bacteria (Figure 4). Bacteria were also seen closely attached to cell membrane of endothelial cell without any structure changes to the cell membrane. The capillaries of the trachea, bacteria in the blood vessel lumen appear to attach to long thick electron dense pedestal like lesions (Figure 5). Attached bacteria to short pedestal were also seen. But this pedestal was also vesicular beside increased in electron density. Beneath this pedestal in the cytoplasm, long, thick, and linear electron dense structure were observed (Figure 5). Bacteria attaching to electron- lucent pedestal were also seen in the trachea (Figure 6). Focal increase in electron density was only observed beneath this pedestal.



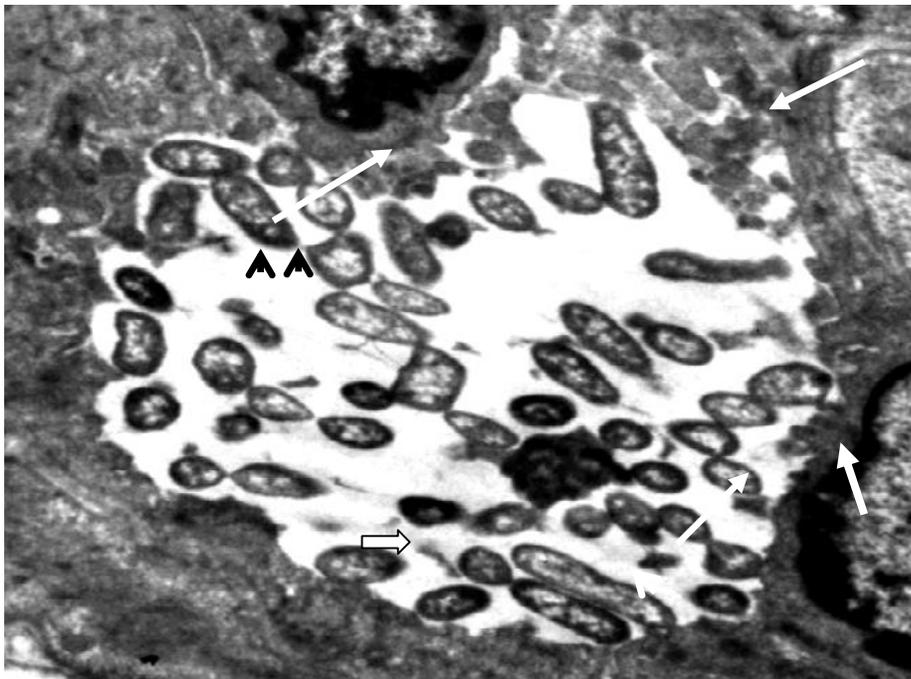
**Figure 1:** Electron micrograph of the trachea of a mouse from group 3 that died 18 hours p.i. Note bacterial cells (double arrow) are associated with the deformed and broken cilia. The outline of the deformed cilia is very electron dense. A bacterium is undergoing division (arrow). Lead citrate & uranyl acetate. TEM X 8000.



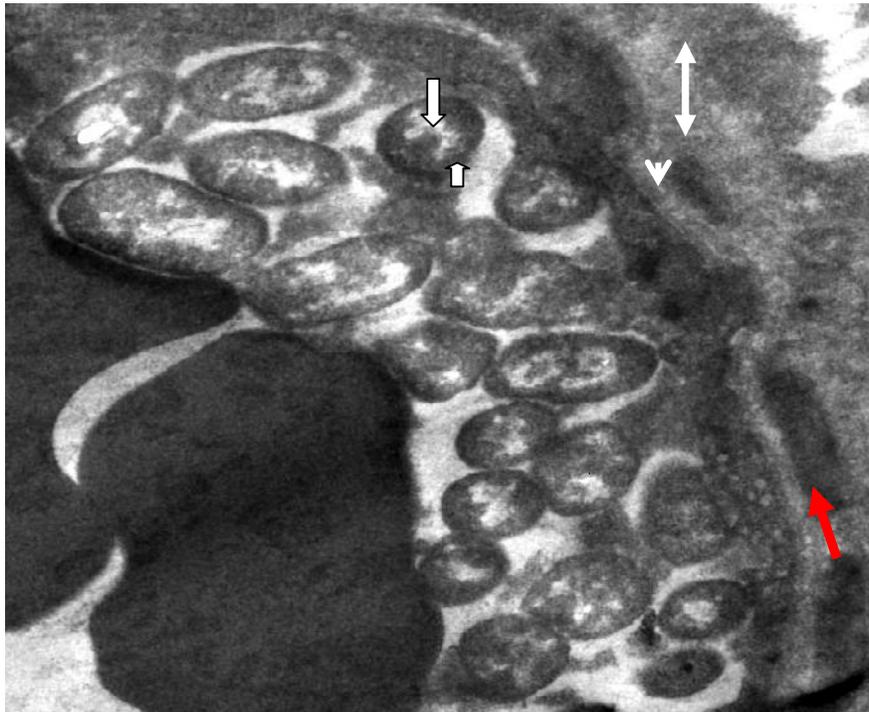
**Figure 2:** Electron micrograph of the trachea of mouse from group 5 that died 22 hours p.i. Note that the bacteria (short arrow) is attached to the enlarged, deformed and shortened cilia. The outlines of the deformed cilia is electron dense (long arrow). Lead citrate & uranyl acetate. TEM X 4500.



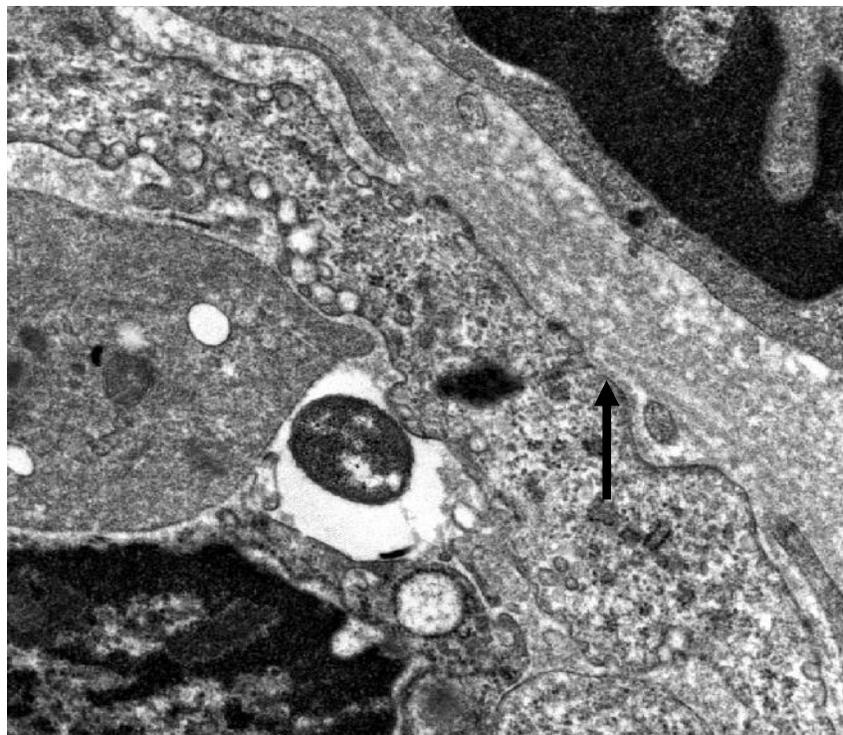
**Figure 3:** Electron micrograph of a capillary in the trachea of a mouse from group 3 that died 18 hours p.i. Note the endothelial cells are swollen with condense cytoplasm (big arrow). Erythrocytes filled the lumen and some are closely adhered to the endothelial cells. Many bacteria are seen in close association with the plasma membranes of the endothelial cells (small arrow). Invagination with slight finger like cytoplasmic extensions (arrow head) appears to engulf the attaching bacteria. Large halos are also seen around the bacteria. The tissues around the capillary contained edema fluid (white arrow). Inset (X 3074) (top left) shows enlargement of the attaching bacteria. Lead citrate & uranyl acetate. TEM X 1537.



**Figure 4:** Electron micrograph of the trachea of a mouse from group 4 that died 20 hours p.i. Note the bacteria in close attachment (block arrow) with the disrupted plasma membrane of swollen endothelial cells in the lumen of trachea capillary. The endothelial cell membranes invaginate with two finger like cytoplasmic extension at the site of the bacteria attachment (long arrows). Single long cytoplasmic extension in a process of engulfing a bacterium is also observed (arrow heads). Lead citrate & uranyl acetate. TEM X 2140.



**Figure 5:** Electron micrograph of the trachea capillary of a mouse from group 4 that died 20 hours p.i. Note many bacteria are seen closely attached to pedestal – like lesion (attaching and effacing lesions) which are either short or long. The pedestal – like lesions which are the extension of the cytoplasm has many features; they are either electron dense (arrow head) or electron dense and vesicular (red arrow). Beneath and adjacent to the pedestal – like lesions are multi foci increased in electron density of the cytoplasm (double arrow). Long, single thin finger-like cytoplasmic extension is also observed surrounding a bacterium (block arrow). Lead citrate & uranyl acetate. TEM X 4000.

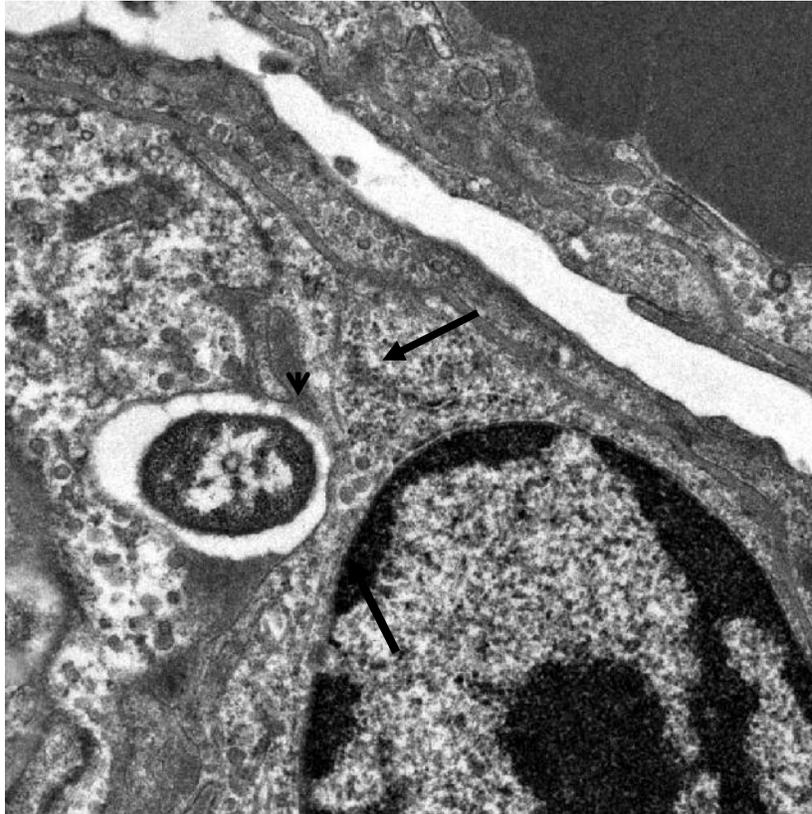


**Figure 6:** Electron micrograph of the trachea capillary of a mouse from group 4 that died 20 hours p.i. Note a bacterium attaches to a pedestal – like lesion of the cytoplasm. Note that the pedestal is not electron dense but a focus of cytoplasmic increased in electron density present beneath to the pedestal (arrow). Lead citrate & uranyl acetate. TEM X 3074.

### 3.3 Lung

Mice in infected groups had congestion of the alveolar capillaries, swelling of capillary endothelium. Numerous bacteria were evident intracellularly and extracellularly. Bacteria was evident close to the

surface of apoptotic pneumocyte. The cell membrane of the apoptotic endothelial cell adjacent to the attached bacteria appeared depressed / invaginated. On the surface of the cell on both sides of the attached bacterium, an electron dense fuzzy material was observed (Figure 7).



**Figure 7:** Electron micrograph of the lung of a mouse from group 3 that died 18 hours p.i. Note the bacteria (arrow head) surrounded by a clear halo is evident close to the surface of apoptotic pneumocyte. The cell membrane of the apoptotic pneumocyte adjacent to the attached bacteria appeared depressed / invaginated. On the surface of the cell on both sides of the attached bacterium, an electron dense fuzzy material is evident (arrows). Lead citrate & uranyl acetate. TEM X 3074.

## 4. Discussion

The ultrastructural observations in this study showed the ability of *P. multocida* to successfully establish and maintain colonization in the respiratory tract. The cytoplasm of the endothelial cells was seen to extend into the lumen, like two short cytoplasmic extensions, single long cytoplasmic extension or long thick electron dense pedestal like lesions. To our knowledge, there has been no report on ultrastructural changes of endothelial cells and cilia due to adhesion of *P. multocida* B:2. This study demonstrated that the adherence of *P. multocida* to the cilia of respiratory tract can induce ciliary degeneration. We thus suggest that ciliostasis as a result of ciliary degeneration occurs very early during *P. multocida* infection and

that this prevents clearance of the bacteria. Deciliation and also stagnation of mucus can promote attachment and colonization of *P. multocida* A:3 in rabbits with subsequent severe damage to the tissues. Our study suggested that *P. multocida* invade animal cell by triggering actin rearrangement that ultimately result changes in the cell shape (during formation of pseudopods (finger like projection) with resulting engulfment. Extensive rearrangement of host cell actin and also condensation of filamentous actin into microfilaments occurs beneath the site of adherence into cube like pedestal structure under the bacteria. Our observation of increased in electron density beneath the point of *P. multocida* suggest that during infection, *P. multocida* induces cytoskeletal changes, by accumulation of polymerized actin directly beneath

the adherent bacteria. The reorganization of actin forms a pedestal-like structure upon which the bacterium resides. The observation of different cell membrane and cytoplasmic changes at sites of *P. multocida* adherence suggests that *P. multocida* may use several mechanisms to hijack host cell signaling machinery and disrupt the cytoskeleton. It seems the involvement of microfilament rearrangements of the cytoskeleton was to facilitate entry of the bacteria to the host cell. *Salmonella* spp. force host cell to engulf them by causing deformation of the cell membrane in the form of ruffling. Ruffling is followed by internalization of the bacteria by extensive actin rearrangement (Salyers and Whitt, 2005). Enterohemorrhagic *Escherichia coli* (EHEC) cause focused actin accumulation beneath the site of bacterial attachment. Following initial adherence to the epithelial cells, EHEC secrete virulence factors, Esps (E. coli-secreted proteins), via a specialized type III secretion system. Secretion of the EHEC virulence factors leads to effacement of the microvillus structure and reorganization of the actin cytoskeleton to form a pedestal-like structure, which is also called the attaching and effacing lesion (Campellone and Leong, 2003). For bacterial pathogens, successful adherence is usually a necessary pre requisite for colonization and even infection. Once adhered to a host surface, some pathogens gain deeper access into the host to perpetuate the infection cycle (Beachey, 1981; St. Geme, 1996). Bacterial attachment to host epithelial cells is mediated by specific interactions between microbial adhesins and complementary receptor structures on the epithelial cell surface (St. Geme, 1996). These adhesions are used by the organisms in the early events in the infection process to attach to specific receptors on mucosal surface (Beachey, 1980). Once access inside the cell, the bacteria is sequestered from host defenses and antimicrobial substances present on the mucosal surfaces. Thus, the microorganism can grow in cytoplasm with nutritionally rich environment free of internal or external interference (Moulder, 1985; Finlay and Falkow, 1988).

In our study, it became apparent that the target cells for *P. multocida* are the endothelial cells of small blood vessels. It seems that adhesion to and invasion of endothelial cell by *P. multocida* are essential for their dissemination throughout the body via blood stream thus causing septicaemia seen in haemorrhagic septicaemia. Relatively little is known about *P. multocida*-host cell interactions. *P. multocida* type A attached specifically to squamous epithelial cells of pharyngeal mucosa of rabbits *in vivo* and *in vitro* and

to some tissue culture cells lines such as Hela (Glorioso *et al.* 1982). The mechanisms of adherence of *P. multocida* to these cells were related to recognition of these bacterial polysaccharides by specific glycoprotein receptors (Glorioso *et al.*, 1982). They also reported that the fimbriated strains of *P. multocida* were more adherent to pharyngeal and HeLa cells than the non fimbriated strains. *Pasteurella multocida* type A was isolated only from lungs of pigs suggesting that type D organism cannot efficiently colonize the lung tissue (Pijoan and Trigo 1990). The same workers suggested that the different pattern of lung colonization may be due to the presence of hyaluronic acid capsule in serotype A, which is not present in serotype D (Pijoan and Trigo, 1990). Thus only *P. multocida* type D cause septicaemia in rabbits but not *P. multocida* type A (Al-Haddawi *et al.*, 1999). *In vitro* adherence of *P. multocida* type D to porcine tracheal rings and high level of bacterial colonization was maintained for at least 24 hours especially after pre-infection with *Bordetella bronchiseptica* (Dugal *et al.*, 1992). These findings indicated that *B. bronchiseptica* facilitated upper respiratory tract colonization by *P. multocida*.

*Candida albicans*, an opportunistic microorganism causing systemic disease, penetrate the endothelial barrier by disrupting cell surfaces soon after attachment, possibly through a process dependent on tissue enzymatic degradation (Klotz *et al.*, 1983). Despite the outward differences between each mode of entry of *P. multocida* into the host cells, it has been believed that *P. multocida* effectively managed to subvert the host cytoskeleton for its own purposes and cause the disease. These results warrants on further studies the pathogenesis of *P. multocida* B:2 infection from adherence to cellular events of host cell invasion.

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